MODIFIED MICROORGANISMS FOR ANTI-CANCER THERAPY

Background of the Invention

In general, the present invention relates to anti-cancer therapy, particularly for the treatment of solid tumors.

Cancer accounts for one fifth of the total mortality in the United States and is the second leading cause of death. The most common anti-cancer therapies used for the treatment of solid tumors are chemotherapy and radiation therapy. The effectiveness of these treatments, however, can be limited by the pathophysiologic characteristics of the solid tumor.

Solid tumors have blood vessels that are structurally and functionally abnormal resulting in poorly vascularized regions of the solid tumor and heterogeneous blood flow throughout. This heterogeneity leads to acutely and/or chronically hypoxic and acidic regions in the tumors which results in large areas of necrosis at the center of the tumor. The ability of chemotherapy and radiation therapy to successfully target the entire tumor is hindered by the inadequate vascularization of the tumor and the presence of the hypoxic center. Chemotherapeutic agents rely on blood vessels for delivery to the tumor cells and therefore are unable to reach the poorly vascularized regions of the tumor. Radiation therapy requires oxygen to impart its cytotoxic effects and is ineffective at killing the cells within the hypoxic center of the tumor. The inability of these therapies to kill all of the cells in the tumor presents a significant limitation to their clinical effectiveness.

One other experimental approach to cancer treatment is the use of anaerobic bacteria to target the necrotic region of the tumor. Such "bacteriolytic therapies" exploit the fact that these necrotic regions are characterized by a hypoxic core where the anaerobic bacteria can preferentially localize and proliferate. However, previous attempts at using these therapies to treat tumors in both mice and humans have shown limited clinical success.

This limited success is due to the inability of these bacteria to disperse in a more homogeneous manner throughout the entire tumor thereby resulting in large colonies of bacteria localized in the necrotic center. Several mechanisms have been proposed to explain this localization to the necrotic core including a specific attraction to the necrotic tissue, an inability to adhere to tumor vasculature, and a limited motility within the tumor itself.

Although significant progress has been made in the development of anti-cancer therapies, and specifically the use of anaerobic bacteria as such an anti-cancer therapy, there are still significant limitations to the currently available treatments which prevent them from becoming clinically effective tools for the eradication of solid tumors.

Another experimental approach utilizes viruses to target neoplastic cells. Proposed viral cancer therapies include two distinct approaches: (i) direct cell killing (oncolysis) by a mutagenized virus and (ii) the use of viral vectors to deliver a transgene whose expression product activates a pathway or an agent that induces cell death. In methods using viral oncolysis, the genetic engineering of viruses for use as oncolytic agents initially focused on the use of replication-incompetent viruses. This strategy was used in an attempt to prevent damage to non-tumor cells by the viruses. A major limitation of this approach is that these replication-incompetent viruses require a helper virus to be able to integrate and/or replicate in a host cell. Therefore, the virus cannot spread far from the producer cell, and are unable to completely penetrate a deep, multilayered tumor *in vivo*.

More recently, genetic engineering of oncolytic viruses has focused on the generation of "replication-conditional" or "replication selective" viruses, which are designed to preferentially replicate in actively dividing cells, such as tumor cells. Replication-conditional viruses have been used in an attempt to avoid systemic infection, while allowing the virus to spread to other tumor cells. Two of the most actively investigated viruses for oncolytic therapy are adenovirus and herpes simplex virus (HSV). However, these replication-

conditional viral mutants can have drawbacks such as undesired viral spread and residual virulence, spontaneous regeneration of the wild-type viral gene, which would render the virus replication competent in normal cells, and limited effectiveness due to tumor cell heterogeneity for the cellular factor(s) necessary to complement the deficiencies of the viral mutant.

As mentioned above, the second approach in viral cancer therapy is the viral delivery of anticancer transgenes such as suicide genes (e.g., thymidine kinase). This type of viral therapy is also subject to many limitations including limitation of viral spread to the region surrounding the producer cell implant, possible immune response to the producer cell line, possible insertional mutagenesis and transformation of retroviral infected cells, and limitation of the bystander effect to cells in direct contact with retrovirally transformed cells.

Although recent clinical trials demonstrate the potential of both oncolytic viruses and viruses carrying transgenes in cancer therapy, the issue of poor distribution and access to cells throughout the tumor is in some cases a major limitation of both types of therapies. Therefore, it remains of utmost importance to develop a safe and effective viral mutant for selectively killing neoplastic cells.

There exists a need for improved viral and bacterial based anti-cancer therapies that can both target neoplastic cells specifically and be effectively distributed throughout the tumor.

Summary of the Invention

As described in more detail below, the invention features microorganisms that are useful as cancer therapeutics and methods of using these microorganisms for the treatment of cancer (e.g., solid tumors). Microorganisms of the invention express recombinant proteins that degrade the interstitial matrix or target the tumor vasculature and enhance the microorganism's ability to disperse throughout the tumor.

In one aspect, the invention generally features a pharmaceutical composition containing (i) one or more genetically engineered microorganisms (e.g., virus or bacteria), where the microorganisms express a nucleic acid encoding a protein that breaks down interstitial matrix or targets tumor vasculature; and (ii) a pharmaceutically acceptable carrier.

In a related aspect, the invention features a kit containing (i) one or more genetically engineered microorganisms, where the microorganisms express a nucleic acid encoding a protein that breaks down interstitial matrix or targets tumor vasculature; and (ii) instructions for their use for treating a cancer (e.g., a solid tumor, such as a solid tumor characterized by hypoxia) in a mammal (e.g., a human).

In another related aspect, the invention features a method of treating a cancer (e.g., a solid tumor, such as a solid tumor characterized by hypoxia) in a mammal (e.g., a human). The method includes administering to the mammal one or more genetically engineered microorganisms (e.g., virus or bacteria), where the microorganism contains a nucleic acid encoding a protein that breaks down the interstitial matrix or targets the tumor vasculature, and the administering is for a time and in an amount sufficient to destroy, slow, or arrest the cancer. In one embodiment, the method also includes administering a therapy selected from any one or more of the group consisting of a chemotherapeutic agent, radiation therapy, an anti-angiogenic compound, an anti-vascular agent, and an oncolytic virus.

In preferred embodiments of any of the above aspects, the nucleic acid encodes a protein selected from the group consisting of a matrix degrading protein, matrix metalloproteinases (MMPs), a protein that increases MMP production, a protein that increases collagen turnover, a protein that decreases collagen formation, a protein that increases extracellular matrix (ECM) turnover, a protein that decreases ECM formation, relaxin, collagenase, anti-fibrotic proteins, halofuginone, hyaluronidase, chondroitinase, heparatinase, and a cathepsin enzyme.

In preferred embodiments of any of the above aspects, the virus is a replication defective virus (e.g., an amplicon viral vector), a replication selective virus, a replication competent virus, or an oncolytic virus. In other preferred embodiments, the virus is a member of a virus family selected from the group consisting of: herpesviruses, adenoviruses, adeno-associated viruses, lentiviruses, parvoviruses, papovaviruses, poxviruses, hepadnaviruses, alphaviruses, iridoviruses, and retroviruses. Most preferably the virus is a herpes simplex virus-1 comprising a mutation in a ribonucleotide reductase gene, wherein the mutation results in inactivation of the ribonucleotide reductase gene; or an adenovirus comprising a mutation in the E1A CR-2 gene, wherein the mutation results in inactivation of the E1A CR-2 gene.

In preferred embodiments of any of the above aspects, bacteria are selected from the group consisting of: *Salmonella bacteriophage*, *S. bongori*, *S. choleraesuis*, *S. enterica*, *S. enteritidis*, *S. paratyphi*, *S. typhi*, *S. typhimurium*, *S. typhimurium bacteriophage*, *Shigella boydii*, *S. dysenteriae*, *S. flexneri*, *S. sonnei*, *Staphylococcus arlettae*, *S. aureus*, *S. auricularis*, *S. bacteriophage*, *S. capitis*, *S. caprae*, *S. carnosus*, *S. caseolyticus*, *S. chromogenes*, *S. cohnii*, *S. delphini*, *S. epidermidis*, *S. equorum*, *S. felis*, *S. fleurettii*, *S. gallinarum*, *S. haemolyticus*, *S. hominis*, *S. hyicus*, *S. intermedius*, *S. kloosii*, *S. lentus*, *S. lugdunensis*, *S. lutrae*, *S. muscae*, *S. mutans*, *S. pasteuri*, *S. phage*, *S. piscifermentans*, *S. pulvereri*, *S. saccharolyticus*, *S. saprophyticus*, *S. schleiferi*, *S. sciuri*, *S. simulans*, *S. succinus*, *S. vitulinus*, *S. warneri*, *S. xylosus*, *Yersinia aldovae*, *Y. bercovieri*, *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, *Y. kristensenii*, *Y. mollaretii*, *Y. pestis*, *Y. philomiragia*, *Y. pseudotuberculosis*, *Y. rohdei*, and *Y. ruckeri*, *Bifidobacterium adolescentis*, *B. animalis*, *B. bifidum*, *B. boum*, *B breve*, *B. coryneforme*, *B. dentlum*, *B. indicum*, *B. infantis*, *B. longum*, *B. magnum*, *B. pseudolongum*, *Lactobacillus bifidus*, *L. delbrueckil*, *Clostridium absonum*, *C. acetobutylicum*, *C. beijerinckii*, *C. bifermentans*, *C.*

butyricum, C. difficile, C. histolyticum, C. novyi, C. oncolyticum, C. pectinovorum, C. perfringens, C. sordelli, C. tetani, C. tyrobutyricum, and Corynebacterium parvum.

In preferred embodiments of any of the above aspects, the nucleic acid encodes a protein selected from the group consisting of a matrix degrading protein, matrix metalloproteinases (MMPs), a protein that increases MMP production, a protein that increases collagen turnover, a protein that decreases collagen formation, a protein that increases extracellular matrix (ECM) turnover, a protein that decreases ECM formation, relaxin, collagenase, anti-fibrotic proteins, halofuginone, hyaluronidase, chondroitinase, heparatinase, and a cathepsin enzyme.

By "cancer" or "neoplasm" is meant an abnormal proliferation of cells, which may be benign or malignant, and which includes solid tumors.

By "solid tumor" is meant a cancer of any tissue other than blood or bone marrow. Solid tumors include, but are not limited to, brain, kidney, liver, nasopharyngeal cavity, thyroid, skin, central nervous system, ovary, breast, prostate, colon, rectum, uterus, cervix, endometrium, lung, bladder, pancreas, and lymph node.

By "viral vector" is meant any replication competent, replication deficient, or replication conditional viral vector that has been modified from the wild-type virus in any way to produce a recombinant virus. Modifications include deletion or mutation of viral genes (e.g., to render the virus replication deficient) or the insertion of a nucleic acid (e.g., a gene or cDNA sequence) encoding a non-viral gene (e.g., a therapeutic gene or a reporter gene). Inserted genes can be operably linked to an endogenous viral promoter or to a separate promoter that is also inserted into the viral vector.

By "amplicon vector" is meant a subset of viral vectors that are replication deficient. Amplicon vectors are composed of an envelope, tegument, and capsid, and a genome that is a linear concatenate of amplicon plasmids. Amplicon plasmids can contain some viral sequences (e.g., the

packaging signal and origin of replication), additional plasmid elements, and a transgene cassette for the expression of a desired gene (e.g., a therapeutic or reporter gene).

By "clonal virus" is meant a virus derived from a single infectious virus particle and for which individual molecular clones have significant nucleic acid sequence homology. For example, the sequence homology is such that at least eight individual molecular clones from the population of virions have sequence homology greater than 95%, preferably greater than 97%, 99%, or 100% over 300 contiguous nucleotides. A clonal virus can be produced according to any method available to the skilled worker. For example, plaque purification is routinely utilized to obtain a clonal virus (see, e.g., Maassab et al., In: Plotkin and Mortimer, eds. Vaccines. Philadelphia: W. B. Saunders Co., pages 781-801, 1994). Triple plaque purification is especially desirable, where a plaque is selected at each round of purification having the desired characteristics, such as a preferred size, shape, or appearance representative of the parental strain. Another means of generating clonal virus is by recombinant DNA techniques known in the art. Another means of obtaining a clonal virus applies the technique of limiting dilution (e.g., by adding dilutions of the virus sample to give an average of one or less infectious virus particles per well containing a monolayer of a susceptible cell). Methods for the purification of clonal viruses are described, for example, in Roberts et al., U.S. Patent Publication No. 20030165465.

By "operably linked" is meant that a gene and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

By "anti-cancer therapy" is meant any therapy that destroys a cancer cell, or slows, arrests, or reverses the growth of a cancer cell. Anti-cancer therapies include, without limitation, radiation therapy, chemotherapy, gene

therapy, anti-angiogenesis therapy, anti-vascular therapy, the therapeutic use of a polypeptide, a virus, an antibody, a liposome, a microorganism, or a combination of any of these therapies.

By "chemotherapeutic agent" is meant a chemical that may be used to destroy a cancer cell, or to slow, arrest, or reverse the growth of a cancer cell. Non-limiting examples of chemotherapeutic agents include, asparaginase, bleomycin, busulfan carmustine (commonly referred to as BCNU), chlorambucil, cladribine (commonly referred to as 2-CdA), CPT-11, cyclophosphamide, cytarabine (commonly referred to as Ara-C), dacarbazine, daunorubicin, dexamethasone, doxorubicin (commonly referred to as Adriamycin), etoposide, fludarabine, 5-fluorouracil (commonly referred to as 5FU), gemcitabine, hydroxyurea, idarubicin, ifosfamide, interferon-α (native or recombinant), levamisole, lomustine (commonly referred to as CCNU), mechlorethamine (commonly referred to as nitrogen mustard), melphalan, mercaptopurine, methotrexate, mitomycin, mitoxantrone, paclitaxel, pentostatin, prednisone, procarbazine, tamoxifen, taxol-related compounds, 6-thiogaunine, topotecan, vinblastine, and vincristine.

By "radiation therapy" is meant the use of directed gamma rays or X-rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that there will be many ways known in the art to determine the dosage and duration of treatment.

By "anti-angiogenic compound" is meant any natural or synthetic compound which prevents the formation of new blood vessels. Anti-angiogenic compounds can be classified as "direct angiogenesis inhibitors," which prevent vascular endothelial cells from proliferating, migrating, or avoiding cell death in response to pro-angiogenic proteins, or "indirect angiogenesis inhibitors," which prevent the expression of or block the activity of a tumor protein that activates angiogenesis (see Kerbel and Folkman, *Nature Reviews Cancer* 2:727-739, 2002 for review and for a list of both direct and

indirect angiogenesis inhibitors). Anti-angiogenic compounds can fall under one or both of the two categories. One example of a direct angiogenesis inhibitor is endostatin, which inhibits endothelial cells from responding to multiple angiogenic proteins. One example of an indirect angiogenesis inhibitor is Iressa (AstraZeneca), which inhibits the synthesis of angiogenic proteins such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and transforming growth factor alpha (TGFα). Anti-angiogenic compounds include, but are not limited to, VEGF inhibitors such as antibodies against VEGF-A, antibodies against one of the VEGF receptors, platelet derived growth factor (PDGF) inhibitors, bFGF inhibitors, placenta-like growth factor inhibitors (PlGF), transforming growth factor beta (TGFβ) inhibitors, interleukin-8 (IL-8) inhibitors, platelet-derived endothelial growth factor (PD-EGF) inhibitors, and small molecule compounds that inhibit the tyrosine kinase activity of one of the VEGF receptors (see Kerbel and Folkman, *supra*). Additional examples of anti-angiogenic compounds include avastin, angiostatin, restin, tumstatin, thrombospondins, canstatin, arrestin, vitaxin, and anti-αvβ3 integrin, as well as other small molecule inhibitors such as TNP-470, 2-methoxyestradiol, and thalidomide. Additional drug compounds with anti-angiogenic activity such as VEGF-Trap, SU6668, SU11248, and Erbitux are also included in this definition. Additional anti-angiogenic drug compounds currently in clinical trials are described in J. Marx (*Science*, 301:452-454, 2003).

By "anti-vascular agent" is meant any agent that specifically targets blood vessels. Preferably, the agent will specifically target tumor blood vessels. It is preferred that anti-vascular agents have a cytotoxic or otherwise anti-cellular effect on the tumor vasculature (e.g., by suppressing the growth or cell division of the vascular endothelial cells). Thorpe *et al.* (*Cancer Res.* 63:1144-1147, 2003) describe two categories of anti-vascular agents: ligand directed, which use antibodies, peptides and growth factors to deliver toxins, pro-coagulants, and pro-apoptotic effectors to the tumor endothelium, and

small molecule compounds, which do not specifically localize to tumor endothelium but exploit pathophysiological differences between tumor and normal endothelium to induce acute vascular shutdown in tumors. Thorpe *et al.* describe numerous examples of each type of anti-vascular agent such as VEGF-gelonin, anti-phosphatidylserine naked antibodies, anti-VCAM1-TF, CA4P, ZD6126, and AVE8062A. All of the exemplary anti-vascular agents described by Thorpe (*supra*) are included herein. Non-limiting examples of anti-vascular agents include antibodies that specifically bind to vascular endothelial cells or proteins produced by vascular endothelial cells, peptides that specifically target blood vessels, and growth factors that bind to vascular endothelial cells or blood vessels. Photodynamic therapy (PDT) is another type of treatment known to have anti-vascular effects (Dolmans *et al.*, *Cancer Res.* 62:2151-2156, 2002). Additional examples of anti-vascular agents can be found in Thorpe *et al.*, U.S.P.N. 6,261,535, incorporated herein by reference.

By "destroy, slow, or arrest" is meant to measurably reduce, stop, or reverse the growth rate of the cancer or cancer cell *in vitro* or *in vivo*. Desirably, the growth rate is reduced by at least 20%, 30%, 40%, 50% or even 70% as determined using a suitable assay for determination of cell growth rates (e.g., *in vitro* cell counting assay, BrdU labeling assay, or *in vivo* tumor size measurement by biopsy or whole body imaging techniques.).

By "interstitial matrix" is meant the stroma that comprises the extracellular milieu. The stroma comprises the interstitial connective tissue, blood vessels, proteoglycans, glycosaminoglycans, collagens, fibrin, connective tissue cells (e.g., fibroblasts), and inflammatory cells.

By "extracellular matrix (ECM)" is meant the matrix that supports the adhesion of cells and transmits signals through cell-surface adhesion receptors. The ECM contains collagens, non-collagenous glycoproteins, and proteoglycans. Alternative ECM components often found in tumors include tenascin, fibronectin, and variant forms of laminin.

By "microorganism" is meant any prokaryotic or eukaryotic single-celled or multi-celled microscopic organism, including, without limitation, bacteria, viruses, and fungi.

By "pharmaceutically acceptable carrier" is meant a carrier that is physiologically acceptable to the treated mammal while retaining the therapeutic properties of the compound with which it is administered. One exemplary pharmaceutically acceptable carrier substance is physiological saline. Other physiologically acceptable carriers and their formulations are known to one skilled in the art and described, for example, in Remington's Pharmaceutical Sciences, (20^{th} edition), ed. A. Gennaro, 2000, Lippincott, Williams & Wilkins, Philadelphia, PA.

By "pharmaceutical composition" is meant a composition that is suitable for administration to a mammal, such as a human. Such a pharmaceutical composition may be prepared according to the techniques known in the art, and administered by any known administration route, for example oral, topical, and injections including but not limited to intravenous, subcutaneous, intramuscular, intratumoral, or intradermal administration.

By "protein that breaks down the interstitial matrix" is meant any protein that can degrade one or more component of the complex three-dimensional network of large macromolecules that comprise the extracellular matrix. Although the specific macromolecules that make up the interstitial matrix can vary, in general, the following macromolecules are found: collagens, non-collageneous glycoproteins, proteoglycans, tenascin, fibronectin, variant forms of laminin, and the epithelial basement membrane. A protein can break down the interstitial matrix by either preventing or reducing the production of or increasing the degradation of any of the macromolecules that make up the interstitial matrix. Desirably, the interstitial matrix is reduced by at least 20%, 30%, 40%, 50% or even 70% as determined using a suitable assay for interstitial matrix degradation. Non-limiting examples of assays for interstitial matrix degradation include *in vitro* invasion

assays using collagen type I, optic nerve explants, fluorikine E assays (R&D Systems) or Matrigel (see Egeblad and Werb (*Nature Reviews Cancer*, 2:161-174, 2002) and references therein); *in vivo* xenograft metastasis assays where tumor cells are injected into the bloodstream of an animal bearing a tumor xenograft, (see Egeblad and Werb, (*supra*) and references therein); imaging of second harmonic generation (Brown *et al.*, *Nature Medicine* 9:796-800, 2003); and histological techniques using chemical staining of collagen (trichrome staining) or immunohistochemistry using antibodies against various types of collagen, proteoglycans (decorin, vesican, lumican), hyaluronanan (hyaluronan-binding protein), and glycoproteins (fibronectin, thrombospondin).

By "protein that targets the tumor vasculature" is meant any protein that specifically recognizes or binds to tumor blood vessels. The proteins can target the vessels by specifically binding to or recognizing a protein expressed by the vascular endothelial cells of the tumor blood vessel. The three categories of proteins that are up-regulated on tumor vessels described by Thorpe *et al.* (2003, *supra*) include (a) molecules associated with angiogenesis and vascular remodeling (e.g., VEGF receptors, fibronectin ED-B domain, $\alpha v\beta 3$ integrins); (b) cell adhesion molecules induced by inflammatory mediators that are secreted by tumor cells and host cells that infiltrated the tumor (e.g., VCAM-1, E-selectin); and (c) molecules associated with prothrombotic changes that occur on vascular endothelium in tumors (e.g., phosphatidylserine). Any protein, antibody, or peptide that specifically targets any of these tumor vessel-specific proteins are included in the definition herein. The ability of a protein to target the tumor vasculature can be assayed by various vascular imaging techniques such as magnetic resonance imaging (MRI), computed tomography (CT), positron emission tomography (PET), ultrasonography, optical imaging, and microscopic methods, including fluorescence, confocal, multiphoton, and electron microscopy (see McDonald and Choyke, *Nature Med.* 9:713-725, 2003).

By "replication defective viral vector" is meant a viral vector that is incapable of independent replication.

By "replication competent viral vector" is meant a viral vector capable of independent replication.

By "replication selective viral vector" is meant a viral vector capable of replication under particular conditions. For example, a replication selective virus may undergo replication in proliferating cells, such as tumor cells, but fail to replicate in non-proliferating cells.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Drawings

Figure 1A shows a transmitted light image of a tumor cylindroid composed of LS174T colon carcinoma cells with a horizontal diameter of 415 μm (white arrow). Figure 1B shows a schematic representation of the constraining environment used to create tumor cylindroids. Figures 1C and 1D show fluorescence images of GFP-expressing *S. typhimurium* (strain SL1344) invading a tumor cylindroid 14 hours after inoculation of the culture medium. Figure 1D shows *S. typhimurium* invading the intercellular space between cells and penetrating into the interior of the cylindroid. The scale bar is 100 μm for Figures 1A and 1C and 25 μm for Figure 1D.

Figure 2A shows the accumulation of *S. typhimurium* (strain VNP20009) into the organs of immuno-competent (C3H) and immuno-deficient (SCID) mice, both with MCaIV murine mammary carcinomas, one week after systemic injection administered at two different doses, 2 million colony forming units (CFU)/mouse and 20 million CFU/mouse. Figure 2B shows the ratio of accumulation in the tumor to the accumulation in the spleen in mice injected with *S. typhimurium* VNP20009, bearing MCaIV carcinomas. Figure 2C shows the ischemic regions (white arrows) in the liver of an immuno-deficient SCID mouse that received 20 million CFU. Figure 2D

shows the spleen mass one week following bacterial (VNP20009) injection in mice bearing MCaIV carcinomas. Figure 2E shows the accumulation of *S. typhimurium*, strain SL7207, in the organs of SCID mice with LS174T colon carcinomas. Significance in all figures was determined using the two-tailed unpaired student's t-test; error bars are ± SEM.

Figure 3A shows *S. typhimurium* (strain VNP20009; observed as small dots in the figure) flowing through the vasculature (visualized with rhodamine dextran-2 million molecular weight) of a SCID mouse bearing an MCaIV tumor that was administered a dose of 20 million CFU. Figures 3B – 3E show the bacterium observed adhering to tumor vasculature (white arrows). Each frame (Figures 3B – 3E) is separated by 33 msec (video rate). This bacterium dislodged from this position after six minutes. The other particles visible in the images are bacteria that adhered previously. Figure 3F shows the representative history of all bacteria that adhered for greater than two minutes within six locations of an MCaIV tumor in a SCID mouse administered 20 million CFU VNP20009. Bacteria either slowly disappeared (shaded bars), were obviously dislodged (terminated solid bars), or persisted until the end of observation (solid bars). The left end of each bar indicates when the bacterium first adhered. Figure 3G shows the flux of bacteria into a location significantly ($p<0.002$) correlated with the number of adherent bacteria that persisted until the end of observation. The scale bars for (A) – (E) are all 100 μm.

Figures 4A-4E show tiled reconstructions of histological sections of Brown-Hopps stained subcutaneous tumors. Figure 4A shows a C3H mouse administered 20 million CFU. Figure 4B shows a C3H mouse administered 2 million CFU, and Figure 4C shows a SCID mouse administered 20 million CFU. The labeled regions are *t* – viable tumor tissue; *n*, – necrotic tumor tissue; and *s* – skin. All labeled colonies were densely packed and typically contained greater than 100 individuals. Sparsely populated tissue contained approximately 1 – 10 bacteria per 290 x 190 μm field of view. No functional vessels were present in the necrotic issue and no bacteria were detected in the

viable tissue. Scale bars are 1 mm. Figure 4D shows an average-sized bacteria colony (white arrows), located at *(*)* in Figure 4A. Nuclear debris from dead tumor cells stained purple (black arrow). Scale bar is 40μm. Figure 4E shows lectin staining used to identify vessels in Figures 4A – 4C. Scale bar is 100μm.

Figure 5A shows a schematic representation of the retinoblastoma (pRb) pathway. During normal cell proliferation, pRb phosphorylation by cyclin-cyclin dependent kinase complexes causes dissociation of pRb from E2F and removal of inhibition. When adenovirus infects normal cells, the viral E1A protein binds and inhibits pRb. In cancer cells, the Rb pathway is defective and E2F is always active. The E1A protein is unnecessary for viral replication in cancer cells.

Figure 5B shows a schematic representation of an exemplary virus used in this invention. The E1A-CR2 deletion in the recombinant oncolytic viruses is a minimal deletion that inhibits binding of the protein to pRb. The E3B gene in the MMP/relaxin oncolytic virus is completely removed and replaced with MMP or relaxin cDNA.

Figure 6 shows a diagram of an HSV-1 amplicon vector plasmid that contains the cDNA for collagenase and DsRed2.

Figure 7 shows a diagram of an amplicon plasmid packaged inside the amplicon vector.

Figure 8 shows a diagram of two of the methods for packaging viral vectors. In each method, the amplicon is first transfected into the packaging cell line. The first method uses a helper virus, which is a defective replication-deficient helper virus lacking essential viral genes and is packaged in a complementing cell line that produces the missing viral proteins. This packaging method generates both the helper virus and the amplicon vector. The second method uses an HSV-1 bacterial artificial chromosome which provides the functions necessary for packaging but lacks packaging signals. Only the amplicon vector is produced using this method.

Figure 9 shows a diagram of DNA replication during viral packaging in cell culture. For HSV-1, rolling circle replication and cleavage occur at the packaging/cleavage sequence resulting in the 150 kB genome that is inserted into the capsid. For the HSV-1 amplicon vector, the amplicon plasmid contains the minimum sequence for packaging, including the origin of replication and the packaging signal. The resulting amplicon vector contains a 150 kB concatamer having multiple copies of the gene of interest.

Figure 10 shows a diagram of the HSV-1 amplicon vector. HSV-1 consists of a lipid bilayer that contains glycoproteins that facilitate binding to the cell surface, a tegument that surrounds the icosahedral capsid, and the double-stranded linear viral genome that is 150 kB in length. The amplicon vector is a replication deficient vector which is similar to HSV-1 except that it lacks the viral genome. Instead, they contain a concatemer (or monomer) that has a packaging signal, an origin of replication, and can have an expression cassette that contains a reporter or therapeutic transgene.

Figure 11 shows a two-photon image of eGFP-labeled HSV injected intratumorally in Mu89 tumor grown in the dorsal skinfold chamber at a depth of 100 to 200 μm from the surface of the tumor. The gray shading depicts collagen imaging by second harmonic generation. Collagen fibers appear to limit viral distribution. Image width is 1.5 mm.

Detailed Description of the Invention

The failure of many current anti-cancer therapies often results from insufficient delivery of therapeutics or other active compounds to the avascular core region of a solid tumor. Microorganisms, such as viruses or bacteria, that are attracted to the tumor cells or that are attracted to the avascular or hypoxic center of the tumor, are useful for treating solid tumors or delivering therapeutics to the inaccessible regions. Because such microorganisms are often unable to move throughout the tumor, an insufficient distribution of the therapeutic agent (i.e., the microorganism) results. We have discovered that

genetically engineered microorganisms that express a protein that targets the interstitial matrix or the tumor vasculature can greatly enhance the ability of the microorganism to disperse throughout the tumor. Such microorganisms can also be used in combination with additional anti-cancer agents to enhance the distribution of the additional agents as well. These microorganisms overcome many of the limitations of current anti-cancer therapies.

Microorganisms

In general, an effective tumor-treating microorganism preferably has any or all of the following characteristics: (1) relatively or completely non-toxic to the host; (2) only able to replicate within the tumor; (3) motile and able to disperse evenly throughout a tumor (including hypoxic and necrotic regions); (4) slowly and completely eliminated from the host; (5) non-immunogenic; (6) easy to genetically modify; and (7) able to cause lysis of tumor cells. These microorganisms typically lyse tumor cells by either (1) direct competition for nutrients, (2) localized production of cytotoxins, (3) production of therapeutic amplifiers, (4) cytopathic effects (CPE) on the host cell, and (5) lysis of the host cell to produce progeny.

The microorganisms used in the current invention include any viruses or bacteria. Preferred examples of each are provided below.

Bacteria

The microorganisms used in the current invention include bacteria, preferably obligate or facultative anaerobic bacteria. Examples of obligate anaerobes include, but are not limited to, *Bifidobacterium adolescentis, B. animalis, B. bifidum, B. boum, B breve, B. coryneforme, B. dentlum, B. indicum, B. infantis, B. longum, B. magnum, B. pseudolongum, Lactobacillus bifidus, L. delbrueckil, Clostridium absonum, C. acetobutylicum, C.*

beijerinckii, C. bifermentans, C. butyricum, C. difficile, C. histolyticum, C. novyi, C. oncolyticum, C. pectinovorum, C. perfringens, C. sordelli, C. tetani, C. tyrobutyricum, and Corynebacterium parvum.

Examples of facultative anaerobes include, but are not limited to, *Salmonella bacteriophage, S. bongori, S. choleraesuis, S. enterica, S. enteritidis, S. paratyphi, S. typhi, S. typhimurium, S. typhimurium bacteriophage, Shigella boydii, S. dysenteriae, S. flexneri, S. sonnei, Staphylococcus arlettae, S. aureus, S. auricularis, S. bacteriophage, S. capitis, S. caprae, S. carnosus, S. caseolyticus, S. chromogenes, S. cohnii, S. delphini, S. epidermidis, S. equorum, S. felis, S. fleurettii, S. gallinarum, S. haemolyticus, S. hominis, S. hyicus, S. intermedius, S. kloosii, S. lentus, S. lugdunensis, S. lutrae, S. muscae, S. mutans, S. pasteuri, S. phage, S. piscifermentans, S. pulvereri, S. saccharolyticus, S. saprophyticus, S. schleiferi, S. sciuri, S. simulans, S. succinus, S. vitulinus, S. warneri, S. xylosus, Yersinia aldovae, Y. bercovieri, Y. enterocolitica, Y. frederiksenii, Y. intermedia, Y. kristensenii, Y. mollaretii, Y. pestis, Y. philomiragia, Y. pseudotuberculosis, Y. rohdei,* and *Y. ruckeri.*

Any of the bacteria listed above can be used in the present invention.

Viruses

The invention also features the use of viral vectors for the delivery of a transgene encoding a protein that targets the interstitial matrix or tumor vasculature. In one embodiment, the virus that expresses the protein is any viral vector or amplicon vector that contains a transgene encoding a protein that targets the interstitial matrix or tumor vasculature. In another embodiment, the virus that expresses the targeting protein is an oncolytic viral vector. In yet another embodiment, the virus that expresses the targeting protein is any viral vector or amplicon vector and is used in combination with an oncolytic virus.

In general, the viruses of the invention include any oncolytic virus, amplicon vector, replication defective virus, replication selective virus, or replication competent virus known in the art. Viruses useful for the invention include RNA viruses (e.g., a cytocidal RNA virus, a single-stranded RNA non-segmented, nonenveloped virus; a single-stranded RNA segmented, enveloped virus; a double-stranded RNA segmented, nonenveloped virus; and a single-stranded RNA non-segmented, enveloped virus) and DNA viruses (e.g., enveloped, double-stranded DNA viruses (including poxviruses); nonenveloped, single-stranded DNA viruses; and nonenveloped, double-stranded DNA viruses).

Preferred viruses include any member of the herpesvirus (e.g., herpes simplex virus-1 and herpes simplex virus-2), adenovirus, adeno-associated viruses, lentivirus, parvovirus, papovavirus, poxvirus, hepadnavirus, iridovirus, and retrovirus families.

Numerous oncolytic viruses are known in the art and are described, for example, in Kirn et al. (1999, In: Gene Therapy of Cancer, Academic Press, San Diego, Calif., pp. 235-248). Oncolytic viral therapy is the use of a modified, replication-competent virus to selectively kill cancer cells (Nettelbeck et al., Sci Am, *289:* 68-75, 2003). Oncolytic adenoviral and HSV vectors have recently been used in clinical trials for the treatment of various cancers (Kirn, Gene Ther, 8: 89-98, 2001; Shah et al., J Neurooncol, 65: 203-226, 2003). Beyond their innate anti-tumor activity, which derives from their ability to lyse infected cells, these replication-competent viruses also possess important transport advantages over replication-defective viruses. Ichikawa and Chiocca (Cancer Research, 61: 5336-5339, 2001) compared the reporter gene expression of replication-defective and -competent HSV-1 in tumors. Three to fourteen days after intratumoral injection the reporter gene was expressed in 30 to 40% of the tumor volume with a replication-competent HSV-1, whereas only 10% of the tumor expressed the reporter gene with the replication-defective virus. Kurihara *et al.* (J Clin Invest, *106:* 763-771, 2000)

observed a similar phenomenon with an oncolytic adenovirus. Following a single intratumoral injection, reporter gene expression was observed heterogeneously throughout the tumor for oncolytic adenovirus (Ad.DF3-E1), and only along the needle track for replication-defective adenovirus. The modification of collagen structure and synthesis by recombinant proteins expressed using methods of the invention enhance the distribution of both replication-defective and -competent viral vectors.

Non-limiting examples of preferred oncolytic viruses known in the art include strains of herpes simplex virus-1 such as HSV-1716, HSV-3410, HSV-R3616 (in which the gene encoding ICP34.5 is deleted), HSV-R47 (in which genes encoding proteins R3616 and ICP47 are deleted), HSV-G207 (in which genes encoding ICP34.5 and ribonucleotide reductase are deleted), HSV-7020, HSV-NVR10 (in which genes encoding 7020 and ICP47 are deleted), HSV-G92A (in which the albumin promoter is a transcriptional regulated promoter), HSV-3616-IL-4, HSV-3616-UB (in which genes encoding ICP34.5 and uracil DNA glycosylase are deleted), and HSV-hrR3 (in which the gene encoding ribonucleotide reductase is deleted). Non-limiting exemplary strains of herpes simplex virus-2 include strain 2701, strain 2616, and strain 2604 Other oncolytic herpes simplex viruses are described by Post et al., *Curr. Gene Ther.* 4:41-51, 2004. In addition, any virus can be screened to identify viruses that are capable of inducing death of a tumor cell in the subject (e.g., by inducing cytolysis or apoptosis in tumor cells).

Additional non-limiting examples of preferred oncolytic viruses known in the art include strains of adenoviruses such as ONYX-015 (i.e., dl1520), ONYX-411, *dl*922-947 (Johnson et al., *Cancer Cell* 1:325-37 2002), CN706 and CN787; NDV such as 73-T; and vesiculoviruses. Examples of replication defective adenoviruses which are defective for p53 and /or retinoblastoma (Rb) sequestration or degradation and can selectively replicate in cells (e.g., neoplastic cells) lacking p53, pRb, or both (see Figures 5A and 5B) can be found, for example in U.S.P.N. 5,856,181. Additional descriptions of oncolytic

viruses that can be used in the present invention can be found in U.S. Patent Application Publication Nos. 20040146488 and 20030091537, and U.S. Patent No. 6,428,968.

The oncolytic virus of the invention may also comprise an exogenous nucleic acid which contributes to the oncolytic effect of the virus. The nucleic acid preferably encodes an anti-oncogenic or oncolytic gene product. The gene product may be one (e.g., an antisense oligonucleotide) that selectively inhibits the growth or replication of the cell infected by the virus, or it may be one (e.g., thymidine kinase) which exerts a significant bystander effect upon lysis of the cell infected by the virus.

Examples of viruses that are particularly useful for the delivery of a transgene encoding a protein of the invention can be found, for example, in U.S. Patent Application No. 20020031527 and U.S. Patent Nos. 6,602,499. Viruses are highly efficient at nucleic acid delivery to specific cell types, while often avoiding detection by the infected host's immune system. These features make certain viruses attractive candidates as gene-delivery vehicles for use in gene therapies (Robbins and Ghivizzani; Pharmacol Ther. 80:35-47, 1998). Retrovirus, adenovirus, adeno-associated virus (AAV), and herpes simplex virus are examples of commonly used viruses in gene therapies. Each of the aforementioned viruses has its advantages and limitations, and must therefore be selected according to suitability of a given gene therapy (Robbins and Ghivizzani; *supra*).

Engineered viral vectors exploit the natural machinery of the virus to achieve a high efficiency of transfection. Vector pharmacokinetics have been acknowledged as a significant factor in gene transfer efficiency, but few studies exclusively address delivery or the microscopic spatial distribution of vectors delivered to solid tumors. In the absence of data about penetration, the design process favors large, complex constructs and delivery vectors that may exhibit sub-optimal delivery characteristics. For instance, with the relatively small AAV vector the limit of tolerable transgene size at only 4,000 base pairs is

cited as a major disadvantage of its application; however, a small genetic "payload" could eventually be regarded as a necessary concession to obtain enhanced penetration into tissue. To determine the optimal gene delivery vector, the diffusion coefficients and spatial distribution of viruses (such as AAV and HSV-1), which have diameters of about 20 and 150 nm, respectively, may be measured by standard techniques.

Adenovirus is also particularly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range and high infectivity. Both ends of the viral genome contain 100-200 base pair inverted repeats (ITRs), which are cis elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression and host cell shut-off. The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). Recombinant adenovirus can be generated using methods known in the art, such as homologous recombination between shuttle vector and provirus vector. Since the E3 region is dispensable from the adenovirus genome, the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, Biotechnology. 20:363-90, 1992). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury et al., EMBO J. 6:1733-9. 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1

and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. Current studies are underway to reduce the amount of adenoviral DNA that remains in the vector.

Helper cell lines for propagation of adenoviruses may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material used to obtain the conditional replication-defective adenovirus vector for use in the present invention.

Adenovirus growth and manipulation is known to those of skill in the art, and exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, e.g., 10^9 to 10^{11} plaque-forming units per ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal and, therefore, have low genotoxicity to host cells.

In one example, the wild-type adenoviral genome is modified to target cancer cells via a deletion in the E1A CR-2 gene (see Figures 5A and 5B), resulting in a tumor cell-selective replication competent vector. Subsequently, the cDNA encoding a protein that targets the interstitial matrix or the tumor vasculature (e.g., an MMP or relaxin) is inserted into the E3 region in place of the non-essential adenoviral gene E3B. The resulting virus is packaged in the

human embryonic kidney HEK293 cell line and purified by CsCl gradient. Expression of the protein that targets the interstitial matrix or the tumor vasculature (e.g., MMP or relaxin) is confirmed *in vitro* by Western blot and quantified by ELISA.

Herpes simplex viruses (HSV) are also preferred for the delivery of a transgene encoding a protein of the invention. HSV is another virus that has found success as an oncolytic agent. HSV is an enveloped, double stranded DNA virus. As with adenovirus, various methods have been used to confer tumor selectivity on HSV. These methods include both the use of a tissue-specific promoter and the incorporation of deletion mutations to inactivate proteins required for viral replication in normal cells (Miyatake et al., *J. Virol.* 71:5124-32, 1997; Martuza et al., *Science* 252: 854-6 1991). The targets for gene deletion include the ICP34.5 gene, which encodes a protein that suppresses the shutoff of host protein synthesis, and the ICP6 gene, which encodes a ribonucleotide reductase that produces dNTPs needed for viral DNA synthesis (Chambers et al., *Proc. Natl. Acad. Sci. U S A* 92: 411-5, 1995; Chase et al., *Nat. Biotechnol.* 16: 444-8, 1998; Yoon et al, *Faseb J.* 14:301-11, 2000). A multimutated vector, G207, has been developed that incorporates mutations in both the ICP34.5 and ICP6 genes (Mineta et al., *Nat. Med.* 1: 938-43, 1995). G207 has exhibited safety in both pre-clinical and clinical settings and is being developed as a therapeutic for various cancers (Hunter et al., *J. Virol.* 73: 6319-26, 1999; Markert et al., *Gene Ther.* 7: 867-74, 2000; Chahlavi et al., *Neoplasia* 1:162-9, 1999; Oyama et al., *Jpn. J. Cancer Res.* 91: 1339-44, 2000; Cinatl et a., *Cancer. Res.* 63: 1508-14, 2003). Methods for generating a recombinant HSV vector for the delivery of a transgene can be found, for example, in U.S. Patent Application Nos. 20040146488 and 20030091537.

HSV amplicon vectors are also useful in the invention. Amplicon vector plasmids are bacterially produced plasmids containing a col E1 ori (an Escherishia coli origin of replication), OriS (the HSV-1 origin of replication), HSV-1 packaging sequence, the transgene under control of an immediate-early

promoter and a selectable marker (Federoff et al., *Proc. Natl. Acad. Sci. USA* 89: 1636-1640, 1992). The amplicon is transfected into a cell line containing a helper virus (a temperature sensitive mutant) which provides all the missing structural and regulatory genes in trans. Both the helper and amplicon containing viral particles are delivered to the recipient. Additional amplicons that include an Epstein-Barr virus derived sequence for plasmid episomal maintenance can also be used (Wang and Vos, *J. Virol.* 70: 8422-8430, 1996).

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription. The resulting DNA stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. In order to construct a retroviral vector, a nucleic acid encoding a gene of interest is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and packaging components is constructed (Mann et al., Cell 33:153-9. 1983). When a recombinant plasmid containing a cDNA, together with the retroviral LTR and packaging sequences is introduced into this cell line (by calcium phosphate precipitation for example), the packaging sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, Biotechnology 10:493-513, 1988; Temin, Cell Biophys. 9(1-2):9-16. 1986; Mann et al., *supra*). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells. Many examples of the use of retroviruses to achieve transfer into tumor cells are known in the art, including U.S. Patent No. 5,688,773.

Adeno-associated virus (AAV) is another attractive vector system for use in the present invention as it has a high frequency of integration and it can infect nondividing cells, thus making it useful for delivery of genes into mammalian cells in tissue culture (Muzyczka, Curr Top Microbiol Immunol. 158:97-129, 1992). AAV has a broad host range for infectivity (Tratschin et al., J Virol. 51:611-9. 1984), which means it is applicable for use with the present invention. Details concerning the generation and use of rAAV vectors are described in U.S. Pat. Nos. 5,139,941 and 4,797,368.

In one preferred embodiment of the invention, MGH-2, a selectively-replicating HSV-1 vector containing a deletion of both copies of the ICP34.5 gene and insertion of EGFP in the place of ICP6/UL39, is used. ICP6 is the large subunit of ribonucleotide reductase (RR), an enzyme necessary for viral replication. Deletion of ICP6 allows for selective viral replication in proliferating cells that provide the enzyme. ICP34.5 is a virulence factor with several functions, one of which is to prevent shutoff of protein synthesis by the infected cell. The vector was engineered in the lab of E. Antonio Chiocca and is similar ti MGH-1, which has a lacZ insertion rather than EGFP (see Jacobs et al., *J. Virol.* 61: 2983-95, 2001 and Kramm et al., *Hum. Gene Ther.* 8: 2057-68, 1997) and G207 (see Markert et al., *Gene Ther.* 7: 867-74, 2000; Mineta et al., *Nat. Med.* 1: 938-43, 1995; and Sundaresan et al., *J. Virol.* 74: 3832-41, 2000).

Genetic engineering of microorganisms

Microorganisms of the present invention are engineered to incorporate genes for the expression of proteins that facilitate the ability of the microorganism to disperse throughout a tumor. General techniques, as well as particular techniques for the generation of bacteria or viruses expressing transgenes, are described below.

Various standard techniques for creating microorganisms that contain nucleic acid sequences operably linked to a promoter for the expression of a polypeptide encoded by the nucleic acid sequences are known to those skilled

in the art. Generally, the first step requires cloning of the gene encoding the protein of choice by methods such as PCR or restriction enzyme digestion, followed by ligation of the gene into a desired expression vector or plasmid.

The nucleic acid is preferably operably linked to a promoter that is capable of driving expression of the protein in the desired target host cell. Preferably, a high expression promoter is utilized. One example of a suitable promoter is the 763-base-pair cytomegalovirus (CMV) promoter. The Rous sarcoma virus (RSV) (Davis, *et al.*, *Hum. Gene Ther.* 4:151-159, 1993) and mouse mammary tumor virus (MMTV) promoters may also be used. Certain proteins can be expressed using their native promoter. Inducible promoters can also be used. Examples of useful inducible promoters include the tetracycline-inducible promoter or a zinc-inducible promoter. Optimally, an inducible promoter will not allow significant expression in the absence of an inducer and will not require toxic amounts of the inducer to generate sufficient expression in tumor cells. Other useful promoter systems, such as the ecdysone (Palli et al., Eur J Biochem, *270:* 1308-1315, 2003; Wyborski et al., Biotechniques, *31:* 618-620, 622, 624, 2001) or dimerizer (Pollock et al., Curr Opin Biotechnol, *13:* 459-467, 2002) systems are also available. Other elements that can enhance expression can also be included (e.g., enhancers or a system that results in high levels of expression such as a tat gene and tar element). The nucleic acid operably linked to a promoter is typically then gel-purified and the microorganism is transformed using standard methods to generate a novel microorganism that can express the protein of choice.

Exemplary methods for genetically engineering microorganisms can be found in, e.g., Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, 2002; Sambrook and Maniatis, Molecular Cloning, A Laboratory Manual, 3rd ed., Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 2001) and Pawelek *et al.*, U.S.P.N. 6,190,657, incorporated herein by reference.

In one example, a chromosome containing the gene of choice is digested with an appropriate restriction enzyme and the obtained gene fragments are subjected to agarose gel electrophoresis. A fragment containing the gene of choice is separated from the agarose gel and subjected to gel purification using standard methods (Sambrook and Maniatis, *supra*). For the methods and compositions involving bacteria, a plasmid is partially digested with an appropriate restriction enzyme (e.g., EcoRI, HindIII, BamHI, SalI, etc.) and the obtained partially digested fragment is ligated to the above gene fragment by a DNA ligase (e.g., T4 ligase, *E. coli* DNA ligase, etc.) to prepare a recombinant plasmid. Bacteria are then transformed with the recombinant plasmid, and those microorganisms expressing the recombinant plasmid are selected for by standard methods (e.g., using the antibiotic resistance gene present in the plasmid; Sambrook and Maniatis, *supra*).

The vector can be any plasmid that is replicable in transformed cells, but is preferably a plasmid which has a copy number of one to several thousands and contains an antibiotic resistance marker (e.g., ampicillin, kanamycin, and chloramphenicol). Such plasmids are commercially available, or may be prepared from microbial cells containing these plasmids by a conventional method, for example, by the "alkaline lysis method" (Sambrook and Maniatis, *supra*)

Homologous recombination as described, for example, in Low *et al.* (*Nat. Biotech.* 17:37-41), can also be used to generate microorganisms expressing the exogenous gene as part of their genome. A host microorganism having a gene incorporated into the chromosome can also be used. Such a host microorganism is prepared by standard homologous recombination methods known in the art. In one example, an exogenous gene is ligated to a plasmid which contains a transposon and a marker gene. The plasmid is then introduced into the host microorganism by homologous recombination.

Transduction, or gene transfer mediated through bacteriophages, can also be used to generate microorganisms expressing the exogenous gene (see for example, Ausubel *et al., supra* and Sambrook and Maniatis, *supra*).

Microorganisms can also be engineered to decrease or eliminate expression of endogenous toxin genes or to express specific genes such as antibiotic sensitivity genes to allow for eradication of the bacteria after therapy is complete, or fluorescent marker genes to track the bacterial distribution. Examples of methods to generate genetically modified tumor-targeting bacteria lacking toxin genes or expressing viral suicide genes such as the thymidine kinase gene from herpes simplex virus can be found in, e.g., Clairmont *et al.* (*J. Infect. Dis.* 181:1996-2002, 2000), Dang *et al.* (*Proc. Natl. Acad. Sci.* 98:15155-15160, 2001), Low *et al. supra*, and U.S. Patent Nos. 6,080,849 and 6,190,657, incorporated herein by reference. King *et al.* (*Human Gene Therapy* 13:1225-1233, 2002) provides exemplary methods for generating genetically modified tumor-targeting bacteria expressing the cytosine deaminase gene used to convert 5-fluorocytosine to the cytotoxic 5-fluorouridine.

For viruses, the transgene may be inserted into the viral genome by any suitable technique such as homologous recombination of the virus with, for example, plasmid vectors carrying the transgene flanked by viral sequences. The gene may be inserted into the viral genome at any location provided that desired viral replicative or oncolytic properties are retained. Transgenes may be inserted at multiple sites within the virus genome. For example, from 2 to 5 genes may be inserted into the genome. Methods for generating recombinant adenoviral vectors are described, for example, in U.S. Patent No. 6,100,086, 5,585,362, and in Wang, et al., J. Biol. Chem. 273, 2161-8, 1998. Methods for generating recombinant herpes simplex viruses by homologous recombination methods are described, for example, in U.S. Patent Application Publication No. 20030091537.

Methods for packaging and generating recombinant viruses are known in the art and can be found, for example, in Ausubel *et al., supra,* Sambrook and Maniatis, *supra,* Saeki Y et al., (*Improved HSV-1 amplicon packaging system using ICP27-deleted, oversized HSV-1 BAC DNA*, In Viral vectors for gene therapy methods and protocols, Machida CA eds., p. 51-60, Humana Press Totowa, NJ, 2003), and Sena-Esteves et al., (HSV-1 amplicon vectors--simplicity and versatility, *Mol. Ther.* 2: 9-15, 2000). In general, replication defective or selective viruses are packaged in a complementing cell line that expresses the proteins necessary for the replication of the virus. Packaging cell lines are known in the art. A diagram of two methods used for packaging amplicon vectors is shown in Figure 8. Viral vectors, particularly amplicon vectors, can also be packaged with fluorescently labeled tegument protein (e.g., using green fluorescent protein (GFP)) for visualization of the particles themselves. For example, retroviral packaging cell lines have the essential retroviral genes (i.e., *gag, pol* and *env)* integrated separately in its genome. Packaging cells which may be transfected include, but are not limited to, the PE501, PA317, R-2, R-AM, PA12, T19-14X, VT-19-17-H2, RCRE, RCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, Human Gene Therapy 1:5-14 (1990).

Proteins

One of the most significant limitations of current methods of viral and bacteriolytic therapy to treat tumors is the inability of the microorganism to disperse throughout the tumor. The genetically engineered microorganisms of the present invention overcome this limitation by expressing one or more proteins that enhance the distribution of the microorganism throughout the tumor. Generally, these proteins will fall into two categories: (1) proteins that break down the interstitial matrix and (2) proteins that target the tumor vasculature. Expression of proteins that break down the interstitial matrix will prevent the microorganism from becoming trapped in the necrotic core of the

tumors. Expression of proteins that target the tumor vasculature will allow the microorganism to specifically target the blood vessels and spread throughout the growing tumor. The genetically engineered microorganisms of the invention can express one or more of these proteins, or fragments thereof. In addition, one or more microorganisms can be used to simultaneously express more than one type of protein. Microorganisms that express the proteins described herein can be used in combination with additional microorganisms that target and kill tumor cells.

Exemplary proteins that either break down the interstitial matrix already present within the tumor environment, prevent further deposition of matrix components, or both include, without limitation, a matrix degrading protein, matrix metalloproteinases (MMPs), a protein that increases MMP production, a protein that increases collagen turnover, a protein that decreases collagen formation, a protein that increases extracellular matrix (ECM) turnover, a protein that decreases ECM formation, relaxin, collagenase, anti-fibrotic proteins, halofuginone, hyaluronidase, chondroitinase, heparatinase, and a cathepsin enzyme (e.g., cathepsin K).

One exemplary protein that breaks down the interstitial matrix is relaxin. Relaxin is a peptide hormone of the insulin-like growth-factor family that is secreted during pregnancy to induce upregulation of various matrix-degrading enzymes such as matrix metalloproteinases. There are several known members of the relaxin gene family including relaxin 1, relaxin 2, and relaxin 3. The preferred relaxin gene will generally be the relaxin gene native to the species of mammal being treated. However, any mammalian form of the relaxin gene or fragment thereof can be used, including but not limited to human, mouse, rabbit, rat, cat, dog, horse, goat, or primate relaxin. Non-limiting examples of relaxin gene sequences from various mammals include GenBank Accession Nos. AY240029 (rat); NM013413 (rat); AF447451 (human); NM_006911 (human); NM_080864 (human); NM_173184 (mouse); XM_129225 (mouse); and AF233688 (cat). Any of the relaxin gene family members or homologous

genes can also be used. Relaxin gene fragments that encode polypeptides shown to be functional, e.g., in the bioassays of Fei *et al.* (*Biochem. Biophys. Res. Comm.* 170:214-222, 1990) and Kramer *et al.* (*In Vitro Cell. Dev. Biol.* 26:647-656, 1990), and in the mouse pubic symphysis assay (Bullesbach and Schwabe, *Biochemistry* 25: 5998-6004, 1986) may also be used for generating the genetically engineered microorganism of the invention. In addition, relaxin gene analogs which encode polypeptides that differ from a native relaxin polypeptide only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, aspartic acid for glutamic acid, etc.) are also included.

Anti-fibrotic proteins include any protein that reduces or inhibits the production of extracellular matrix components including, but not limited to, fibronectin, proteoglycan, collagen, and elastin.

MMPs are another class of exemplary proteins of the first category which may be expressed by the genetically engineered microorganisms of the invention to enhance the motility of the microorganism. MMPs comprise a family of zinc-dependent endopeptidases capable of degrading ECM macromolecules. The MMPs constitute a family of over 24 members, which collectively are capable of degrading virtually the entire ECM. Structurally, MMPs contain a zinc (II) ionic site at the active site of the protein. Binding of zinc to the ionic site is required for hydrolytic activity. A detailed list of MMPs, including MMPs 1-3, 7-9, and 10-28, can be found in Egeblad and Werb (*supra*) and the supplementary table associated with this review. Included in this list of MMPs are various forms of collagenase enzymes such as collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-4 (MMP-18). Preferred MMPs include MMP-1, 8, and 13. The list of MMPs provided by Egeblad and Werb is not intended to limit the invention but to provide a compilation of recent information regarding the MMP family.

The second category of proteins used to create genetically engineered microorganisms of the present invention includes proteins that target the tumor vasculature. Tumor vasculature is morphologically abnormal and various cell-surface and ECM proteins can be used as markers to distinguish tumor vessels from normal vasculature. Expression of proteins that target the tumor vessels by the microorganisms of the present invention can help them to adhere to tumor vasculature and to spread more uniformly throughout the tumor environment.

The proteins can target the vessels by specifically binding to or recognizing a protein expressed by the vascular endothelial cells of the tumor blood vessel. The three categories of proteins that are up-regulated on tumor vessels described by Thorpe *et al.* (2003, *supra*) include (a) molecules associated with angiogenesis and vascular remodeling (e.g., growth factor receptors, fibronectin ED-B domain, αvβ3 integrins); (b) cell adhesion molecules induced by inflammatory mediators that are secreted by tumor cells and host cells that infiltrated the tumor (e.g., VCAM-1, E-selectin); and (c) molecules associated with prothrombotic changes that occur on vascular endothelium in tumors (e.g., phosphatidylserine). Additional non-limiting examples of tumor vessel specific proteins can be found in Ruoslahti (*Nature Rev. Cancer* 2:83-90, 2002) including integrins such as αvβ 3 and αvβ 5, tyrosine kinase receptors such as the VEGF family of receptors, the ephrin family of receptors, MMPs, aminopeptidase N (APN), and endoglin, a cell-surface protein that binds transforming growth factor β. Additional proteins known to be involved in tumor vessel growth include VEGF, bFGF, PlGF, PDGF, IL8, TGF-β, and PD-EGF (see Kerbel *et al.*, *supra* for review). Any antibody, protein, or peptide that specifically targets any of these tumor vessel specific proteins can be used to create genetically engineered microorganisms of the present invention. In one example, a microorganism can be genetically engineered to express a gene encoding VEGF or a VEGF family member which would target and bind to the VEGF receptor expressed on the tumor

vessel. In another example, a microorganism can be genetically engineered to express a gene encoding an antibody or an antigen-binding fragment that specifically binds to the αvβ3 integrin expressed on the tumor vessels.

In addition, methods used to identify peptides that home to tumor blood vessels can also be used to identify peptides that are useful for the present invention. In one example, Ruoslahti (*supra*) describes a technique using phage display libraries *in vivo* to select for peptides that specifically target tumors and tumor vessels in a mouse tumor model. The target tumor is then isolated and put through subsequent rounds of selection to further enrich for the tissue-specific phage. The DNA encoding the tumor vessel specific peptide is then isolated, sequenced, and used to create the genetically engineered microorganism of the invention.

Methods for assaying microorganisms for protein expression and therapeutic effectiveness

Genetically modified microorganisms of the present invention may then be tested for their ability to express the exogenous genes or proteins using any standard methods for protein expression known in the art. Non-limiting examples of protein expression assays include SDS-PAGE followed by Coomassie blue or silver staining of the proteins in the gel, western blotting, and immunoprecipitation. Gene expression can be measured using standard methods for RNA analysis such as northern blotting, PCR based amplification, or RNAse protection assays.

In addition, expression of the exogenous gene or protein can be measured after administering the microorganism to a subject. For example, bodily fluids, such as blood, serum, or urine, from the subject can be measured for expression of the protein or the gene. Methods used to measure levels of proteins in bodily fluids include ELISA, western blotting, or immunoassays using specific antibodies. Methods used to measure gene expression in bodily

fluids include the preparation of RNA from samples taken from the subject and the use of the RNA for northern blotting, PCR based amplification, or RNAse protection assays.

Once it is determined that the genetically engineered microorganism stably expresses the gene or protein of choice, the ability of the microorganism to kill tumor cells can be tested *in vitro* and *in vivo*.

The ability of the microorganism to successfully kill the tumor cells and reduce the size of the tumor can be measured using known imaging techniques (e.g., X-ray, CT scan, MRI, PET, and single photon emission tomography (SPECT)) or biopsies of the tumor itself.

Intravital microscopy (IVM) is another imaging technique that is very useful for monitoring delivery, distribution, and therapeutic efficacy of the microorganisms of the invention (Jain *et al. Nature Reviews Cancer* 2:266-276, 2002). This technique allows for a temporal visualization of bacteria after injection as they move through the vasculature to the tumor site. Intravital microscopy also allows for visualization with resolution all the way down to the subcellular level. IVM requires an appropriate animal model, a molecular probe, a microscope equipped with a digital camera detection system, an image acquisition system, and a computer to process and analyze the data. Each of these requirements is discussed in detail in Jain *et al., supra*.

Imaging techniques (e.g., second-harmonic generation (SHG) with out without circularly polarized light; Brown *et al., supra*) can also be used to monitor the ability of the microorganism to successfully modify the extracellular matrix and disperse throughout the tumor. The SHG imaging results will be confirmed by immunohistochemistry and chemical assays or by polarization microscopic analysis of tissue sections stained with picrosirius red to provide information on collagen fiber thickness (thin versus thick fiber).

The ability of the microorganism to invade and successfully disperse throughout the tumor can also be measured using *in vitro* tumor models such as tumor spheroids (Sutherland, *Science*, 240:177-184, 1988), or tumor

cylindroids. Cylindroids have similar geometric properties to tumor spheroids; they contain rapidly proliferating cells in the outer layer, necrotic centers, and a quiescent boundary region. However, cylindroids have optically accessible cores, and are therefore better suited for microscopically observing the penetration of microorganisms deep into tissue (>100 μm). In addition, because the interior of cylindroids can be monitored in real time, bacterial motion can be observed within a growing mass of cancer cells.

An animal tumor model can be used as an *in vivo* model to determine the ability of the microorganism to invade and successfully disperse throughout the tumor. There are many animal models for a variety of tumors including solid tumors such as brain tumors, prostate tumors, and mammary tumors. One exemplary model is a mouse tumor model in which dorsal skinfold chambers are surgically implanted into either severe combined immuno-deficient (SCID) mice or immuno-competent C3H mice (male, ~30g body weight), as described by Leunig *et al.* (*Cancer Research*, 52:6553-6560, 1992). After a two-day recovery period, small pieces (~1 mm^3) of MCaIV murine mammary carcinomas are implanted into the chambers and tumors are monitored in the dorsal skin fold chambers. Microorganisms of the invention can be injected into the model animals once the tumors have reached a pre-determined size (e.g., 4 to 7 mm). Distribution of the microorganisms can be measured by quantification of bacterial or viral accumulation in the tumor and other organs as described herein. Overall levels of virus in the tumor, its distribution, and its effect on tumor size can also be measured at various time points by harvesting tumors, releasing virus, and titering the virus. In addition, immunostaining can be performed to label both the virus and the expressed protein of choice. Viral spread and protein expression in the tumor can be observed and compared.

The activity of MMPs (e.g., MMP-1, -8 or –13) in tumor extracts can also be measured by fluorokine E assays (R&D Systems). In control experiments known concentrations of the relevant MMP (activated form) will be added to the tumor extract to test the reliability of the fluorokine E assays.

The effect of overexpression of MMPs (e.g., MMP-1, -8 or –13) on the degradation of fibrillar collagen can also be assayed. Tumor cells overexpressing MMP are co-polymerized with collagen I containing fluorescent collagen monomers and the fluorescence intensity of the supernatant is measured. The collagenolytic activity of tumor tissue can also be measured by adding tumor extracts to the collagen I gels spiked with fluorescent collagen monomers. To control for the activity of other enzyme families in the collagenolytic activity assay, cysteine, aspartic and serine proteases will be inhibited with E-64, pepstatin A and aprotinin, respectively. 1-10-phenanthroline will be used to inhibit MMP activity.

Tumor size is measured during and after the administration of the genetically engineered microorganism and measurements are compared to tumor size measurements taken prior to treatment with the microorganism. Biopsied tumor samples can also be assayed for the presence of bacteria or viruses to determine if these microorganisms successfully target the specific tumor.

Additional exemplary methods for analyzing the biodistribution of the genetically engineered microorganism in mouse tumor models can be found in Clairmont *et al.*, *supra* and Dang *et al.*, *supra.*

Combination therapies

Although the administration of the genetically engineered microorganism to a subject is effective as a treatment for tumors, combination therapies are also included within the invention and may prove to be more effective at complete eradication of the tumor.

Radiation therapy can be used in combination with the methods of the present invention. Radiation therapy includes the use of directed gamma rays or X-rays to induce sufficient damage to a cell so as to limit its ability to function normally or to destroy the cell altogether. It will be appreciated that

there will be many ways known in the art to determine the dosage and duration of treatment. Typical treatments are given as a one time administration and typical dosages range from 10 to 200 units (Grays) per day.

Chemotherapy is another example of an anti-cancer therapy that can be used in combination with the methods of the present invention. Several classes of chemotherapeutic agents are available, and many may be used in combination. Accordingly, it will be understood that where a chemotherapeutic agent is referred to in accordance with the present invention, a combination of two or more such agents may be employed. The agent(s) may be introduced into the body as a whole, or their administration may be concentrated at the tumor site.

Useful chemotherapeutic agents include, without limitation, alkylating agents, nitrosoureas, anti-metabolites, plant alkaloids, anti-cancer antibiotics, and steroid hormones. Each agent is categorized according to its effect on the cell cycle and cell chemistry. Alkylating agents, for example, are useful chemotherapeutics that kill cells by directly attacking DNA, and may be used, according to the invention, in the treatment of, for example, lymphomas, and certain carcinomas of the lung, breast, prostate, and ovary. One commonly used alkylating agent is cyclophosphamide.

Nitrosourea drugs are also useful chemotherapeutics of the invention which, being able to cross the blood-brain barrier, may be used, for example, to treat brain tumors, as well as lymphomas, multiple myeloma, and malignant melanoma. Drugs of this category, to which carmustine (BCNU) and lomustine (CCNU) belong, act similarly to akylating agents and, additionally, inhibit changes necessary for DNA repair.

Another category of chemotherapeutics useful in the present invention is the anti-metabolite category, which includes drugs that block cell growth by interfering with certain activities during the "S" phase of the cell cycle, usually DNA synthesis. Once ingested into the cell, anti-metabolites halt normal development and reproduction, and are useful, for example, for the treatment of

choriocarcinoma, and tumors of the gastrointestinal tract, breast, and ovary. Examples of commonly used anti-metabolites are 6-mercaptopurine and 5-fluorouracil (5FU).

Plant (vinca) alkaloids are plant-derived anti-tumor agents which may also be exploited in the methods of the invention, and include vincristine and vinblastine. These agents, which act specifically by blocking cell division during mitosis, are commonly used in the treatment of Hodgkin's and non-Hodgkin's lymphomas, neuroblastomas, Wilms' tumor, and cancers of the lung, breast, and testes.

Anti-cancer antibiotics are another diverse group of compounds that may be used in the methods of the invention and that, in general, act by binding with DNA and preventing RNA synthesis. These agents may be used for the treatment of a variety of cancers, and include doxorubicin (Adriamycin), mitomycin-C, and bleomycin.

Steroid hormones, or hormone antagonists, may also be used as chemotherapeutic agents, given their abilities to modify the growth of certain hormone-dependent cancers. This class includes adrenocorticosteroids, estrogens, anti-estrogens, progesterones, and androgens. One example of a steroid hormone antagonist is tamoxifen, a drug used for estrogen-dependent breast cancer.

Therapeutic antibodies (e.g., Herceptin) can also be used for combination therapies of the invention. Additional therapeutic compounds such as NSAIDS, aspirin, Cox inhibitors such as celecoxib can also be used for combination therapies of the invention.

In addition to the above, any other chemotherapeutic agent may be used in the methods of the invention, including other anti-cancer agents whose mechanisms of action do not permit broad categorization.

In one embodiment of the invention, the combination of a virus containing a transgene encoding a protein that targets the interstitial matrix or the tumor vasculature (either the oncolytic virus or the replication defective

virus or amplicon) is used to improve the diffusion and thereby the efficacy of a small molecule chemotherapeutic (e.g., taxol) or an antibody-based cancer drug (e.g., Herceptin). In this example, Herceptin and taxol are delivered by intravenous injection, and the virus is delivered intratumorally.

In another embodiment, a virus containing the transgene encoding a protein that targets the interstitial matrix or tumor vasculature is administered in combination with an oncoloytic virus. In this embodiment, the virus containing the transgene is used to enhance the diffusion of the oncolytic virus throughout the tumor.

Therapeutic treatments

The microorganisms of the present invention can be administered systemically, locally, or both. Preferable routes of administration include, for example, oral, topical, and injections including but not limited to intravenous, subcutaneous, intramuscular, intratumoral, or intradermal. For systemic delivery it is preferred that the microorganisms are injected intravenously or intratumorally. A therapeutic number of colony forming units (CFU) or plaque forming units (pfu) will be determined empirically for each genetically modified organism in order to optimize the cytotoxic effects to the cancer cells while reducing the harmful side effects to the patient. In general, for bacteria, the dosage for a mammal ranges from 1.0 CFU/kg to about 10^{10} CFU/kg; preferably from 10^2 to 10^8 CFU/kg, more preferably from 10^4 to 10^8 CFU/kg. Examples of injection of spores of up to 10^8 CFU *C. novyi* can be found for example in Dang *et al.*, *supra*. Re-application of the microorganism can be utilized to provide additional periods of expression of the therapeutic protein.

For viral-based therapies, therapeutic treatment may be carried out following direct injection of the virus composition into target tissue, such as the tumor or a blood vessel supplying the tumor. The amount of virus administered can range from 10^2 to 10^{10} pfu, preferably from 10^4 to 10^8 pfu, more preferably about 10^6 to 10^8 pfu. Typically up to 1 mL, typically from 1 to

500 µl, preferably from 1 to 100 µl of a pharmaceutical composition consisting essentially of the virus and a pharmaceutically acceptable suitable carrier or diluent is used for injection, but for some oncolytic therapy applications larger volumes of up to 10 ml may also be used, depending on the tumor and the inoculation site.

For combination therapies, a preferable treatment regimen involves initiation of microorganism therapy prior to the anti-cancer therapy (e.g., chemotherapy or radiation therapy) and continuation of both therapies as necessary.

Examples

The following examples describe assays that were used to monitor the distribution of *Salmonella typhimurium* in tumor cells and in the tumor vasculature using both *in vivo* and *in vitro* approaches. These methods can be used to determine the ability of microorganisms of the invention to successfully disperse throughout the tumor. The following examples are shown to illustrate but not limit the scope of the present invention.

Example 1. Invasion of *S. typhimurium* into a three-dimensional tumor model *in vitro*.

A three-dimensional tumor model (tumor cylindroids, Figures 1A-1D) was specifically created to observe the invasion of bacteria into tumor tissue. Cylindroids have similar geometric properties to tumor spheroids (Sutherland, *supra*); they contain rapidly proliferating cells in the outer layer, necrotic centers and a quiescent boundary region. However, cylindroids have optically accessible cores, and are therefore better suited for microscopically observing the penetration of *S. typhimurium* deep into tissue (>100 µm). In addition, because the interior of cylindroids can be monitored in real time, bacterial motion can be observed within a growing mass of cancer cells.

Spheroids were grown in the bottom of a 96-well plate fitted with custom made lids containing cylindrical plexiglass plugs with diameter less than that of the wells. These plugs, when inserted into the wells, defined a gap that constrained the growth of the spheroids. The gap height was kept constant at 150 μm for all cylindroids.

Figures 1C and 1D show the extent that *S. typhimurium* invaded into a cylindroid in 14 hours. Rapidly swimming *S. typhimuria* were clearly capable of penetrating into tumor cylindroids. During this experiment, bacteria were observed "burrowing" between tumor cells by lining up with the intercellular space and propelling themselves forward. These observations demonstrate the ability of *S. typhimurium* to penetrate into a solid mass of tumor tissue and imply that *S. typhimurium* is capable of redistributing within tumors *in vivo*.

Example 2. Tumor specificity and dose dependence of *S. typhimurium* accumulation *in vivo*.

The accumulation of *S. typhimurium* (strain VNP20009) in the organs of mice bearing MCaIV tumors, one week following injection was measured and is shown in Figures 2A-2E. Significantly (>10,000 fold) more bacteria ($p<0.0002$) accumulated in the tumor than any other organ for C3H mice injected with 2 million CFU (Figures 2A and 2B). The spleen accumulated more bacteria than the other organs for most of the mice (two had slightly more in the liver). This supports the findings of Low *et al.* (*Nature Biotechnol.* 17:37-41, 1999), demonstrating a tumor accumulation ratio greater than 2000 for this strain of *S. typhimurium* in mice.

The ratio of bacterial accumulation in the tumor to accumulation in the spleen (generally the organ with the greatest accumulation) was strongly dependent on dose (Figure 2B). Surprisingly, mice injected with 20 million CFU accumulated similar numbers of bacteria in tumors compared to the mice injected with two million CFU. On the other hand, bacterial accumulation in the other organs increased with dose escalation (Figure 2A). This phenomenon

was observed in both immuno-competent C3H mice and immuno-deficient SCID mice, indicating that it is not dependent on a fully functional immune system. Either a maximum bacterial growth rate is achieved in tumors or a threshold for clearance is crossed when bacteria can no longer be cleared from normal organs. Low *et al.* (*supra*) also observed that only a narrow dose range could produce significantly large accumulation ratios.

The higher bacterial dose affected the health of the SCID mice; four of five SCID mice did not survive injections of 20 million CFU. The liver of the surviving mouse (Figure 2C) had obvious infarcts, possibly due to ischemia as a result of bacterial occlusion or aggregates of leukocytes and platelets activated in the presence of the bacteria. The higher dose induced splenomegaly (Figure 2D) in both strains of mice. However, the higher dose did not appear to affect the health of the C3H immuno-competent mice; all survived and were normally active one week after the injection.

The accumulation of *S. typhimurium* strain SL7207, which is also currently being investigated as a tumoricidal agent (Bereta *et al., In* Abstracts for AACR Conference, San Francisco, CA, 2002), was greater in normal organs than the tumors (Figure 2E). However, SL7207 detrimentally affected the health of the mice. Every mouse had splenomegaly, infarcted livers (similar to Figure 2C) and pungent ascites one week after injection with 2 million CFU.

Example 3. Sparse adhesion of bacteria to tumor vasculature.

The delivery of *S. typhimurium* into MCaIV tumors was observed using intravital microscopy (Figures 3A-G). After an injection of 20 million CFU, bacteria could clearly be seen flowing in the blood stream in each observed tumor location (Figure 3A). Numerous bacteria were observed (>3.6 bacteria/sec, maximum 9.2 bacteria/sec) in each 0.59 x 0.44 mm field of view. After an injection of two million CFU, ten times fewer bacteria were observed in each location.

To determine adhesion events, 20 million CFU bacteria were injected. No bacteria adhered to the walls of vessels with high flow rates (defined as greater than ~1.5 mm/sec) in any of the fourteen mice observed. In vessels with lower flow rates, occasional interactions occurred. Figures 3B – 3E show a bacterium adhering; it enters via a small vessel on top of the larger vessels that are more clearly visible in these figures. The bacterium is not present in Figure 3B. In Figure 3C, the moving bacterium appears as an elongated blur as it enters the focal plane. In Figure 3E, it finally comes to rest in the blood vessel. During one hour of observation, no qualitative drop in bacterial flux into each field of view was detected. No bacteria were detectable in the blood 24 hours after injection.

The appearance and disappearance of 10^6 adherent bacteria were tracked in 23 locations of four mice (Figure 3F). Only bacteria that adhered for greater than two minutes were tracked. Each either dislodged, slowly disappeared or persisted until the end of observation. Fluorescence of the bacteria may slowly disappear due to photobleaching, bacterial migration, endocytosis, reduced GFP expression, or GFP diffusion through bacterial membranes with increased permeability. Total flux of bacteria into a location was determined by counting all bacteria that entered the field of view over a given period of time. This overall flux represents the sum of all bacterial fluxes through each of the vessels visible in the field. Only $0.035 \pm 0.015\%$ (or less than 4 in 10,000) of the bacteria that flowed into the tumors permanently adhered (defined as disappearing or persistent bacteria observed for greater than 12 minutes).

On a *per field* basis, the number of adherent bacteria per time (expressed as rate of adhesion) increased with an increasing overall bacterial flux (Figure 3G, $p<0.002$). In other words, the more bacteria that flowed into a location the more that adhered. The dependence in Figure 3G coupled with the observation that bacteria do not adhere to rapidly flowing vessels suggests that bacteria will

preferentially adhere (in terms of bacteria per volume) in tissue that contains numerous slow and tortuous vessels, i.e., tumors. Note that the average residence time of dislodged bacteria was 7.6 minutes.

Example 4. Colonization of *S. typhimurium* in the necrotic regions of tumors.

The location of bacteria that had accumulated in the tumors was determined by Brown-Hopps staining of transverse sections, cut orthogonally from the cover slip in the dorsal-skin-fold chamber (Figures 4A-4E). The same mice were used for intravital microscopy, quantification of bacterial accumulation, and histological sectioning. For each of the sections 100X magnification was used to locate bacteria and identify whether they were in colonies or spread sparsely (Figure 4A–4C). Biotinylated lectin perfusion stained only vessels with active blood flow, allowing identification of functional vasculature (Figure 4E).

No bacteria were found in the living tumor tissue of any of the mice; they were only present in the necrotic regions (Figures 4A – 4C). Bacteria could not be found in smaller tumors that lacked necrosis. Most of the bacteria were found in large colonies (Figure 4D) that occupied a small percentage of the total tumor volume (the sections shown in Figures 4A – 4C contained more bacteria than the sections from the other mice). The average distance between the colonies and the functional vasculature was 750 ± 40 μm with a minimum distance of 100 μm.

These results suggest that *S. typhimurium* have limited motility within the tumor and survive only within the necrotic regions significantly distant from functional vasculature.

The following materials and methods were used in the examples described above.

Bacterial culture

Three strains of *S. typhimurium* were grown in Luria-Bertani (LB) broth and on agar plates using standard procedures. SL1344 and the green fluorescent protein (GFP)-expressing pSMC21 were a kind gift of Dr. Fred Ausubel, Massachusetts General Hospital and Harvard Medical School; SL7207 was a kind gift of Dr. Bruce Stocker, Stanford Medical School; VNP20009 was provided by Vion Corp., New Haven, CN. SL7207 is an *AroA*$^-$ mutant that is less pathogenic in mice than wild-type SL1344 (Hormaeche C.E. *et al.*, *Vaccine* 14:251-259, 1996) and is currently being investigated as a tumoricidal agent (Bereta *et al.*, *supra*). VNP20009 is a *msbB*$^-$ and *purI*$^-$ mutant that was specifically developed as a non-pathogenic, tumoricidal agent (Low *et al.*, *supra*).

Both SL7207 and VNP 20009 were transfected with pSMC21 by electroporation: 25μF, 400Ω and 2.4kV, using 0.2 cm cuvettes. After transfection, all strains were immediately frozen in LB with 10% glycerol and subsequently maintained in culture with 250 μg/mg kanamycin.

Prior to injection, all strains were grown overnight in LB from single colonies on fresh agar plates, sub-cultured 1 in 10, grown to an OD_{620} of 0.5-0.7, centrifuged at 3700 RPM (3200 x g) for 10 minutes and resuspended in sterile PBS.

Mammalian Cell Culture

LS174T colon carcinoma cells were grown at 37°C, 5% CO_2 in DMEM with 10% FBS. Tumor cylindroids were formed by constraining spheroids between two parallel horizontal surfaces with a defined spacing of 150μm (Figure 1A). Cell aggregates were grown in tissue culture flasks coated with 0.5 mg/cm^2 methacrylate (polyheme) for one week to form spheroids. Individual spheroids, 300 – 400 μm in diameter, were then transferred to 96-well plates and a polycarbonate lid with protruding cylindrical pins (± 25 μm in length and 3 mm in diameter) was lowered onto the spheroids (Figure 1B).

Cylindroids were allowed to equilibrate for 24 hours before injection of bacteria into the culture medium (final concentration 1000 CFU/ml) through holes in the polycarbonate lid. Intra-tumor cylindroid invasion was then monitored microscopically for 24 hours.

Animal model

Dorsal skinfold chambers were surgically implanted into either severe combined immuno-deficient (SCID) mice or immuno-competent C3H mice (male, ~30g body weight), under anesthesia (90 mg ketamine / 10 mg xylazine per kg body weight), as described previously (Leunig *et al*, *Cancer Research* 52:6553-6560, 1992). After a two-day recovery period, small pieces (~1 mm^3) of MCaIV murine mammary carcinomas were implanted into the chambers. Tumors were monitored through glass coverslips in the dorsal skin fold chambers. Intravital bacterial delivery experiments began once the tumors reached an *en face* diameter of 4 - 7 mm. All procedures were carried out following the Public Health Service Policy on Humane Care of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Intravital Microscopy (Measurement of bacterial delivery in vivo)

Five minutes prior to bacteria injection, mice were anesthetized (90 mg ketamine / 10 mg xylazine per kg body weight) and injected with 0.05 ml of 5 mg/ml rhodamine dextran (2-million molecular weight; *Molecular Probes,* Eugene, OR), to identify functional vasculature as described previously (Brown *et al., Nat. Med.* 7:864-868, 2001).

Anesthetized mice were restrained and placed on an upright microscope (*Zeiss*, Göttingen) equipped with an automated stage (*Burleigh*, Fischer, NY). Four to seven vascularized regions were identified in each tumor depending on their size and extent of vascularization. The automated stage was programmed to return to these locations and cycle between them every 20 or 30 seconds. Images were acquired through a 20X objective, using a FITC filter set at video-

rate using a Hamamatsu CCD camera and a Panasonic S-VHS video tape recorder. After one complete cycle was recorded, bacteria were injected intravenously at either 2 10^6 CFU or 20 x 10^6 CFU / mouse, suspended in 0.2 ml sterile PBS.

Quantification of Bacterial Accumulation

One week after bacterial injection, the mice were anesthetized and injected with biotinylated *Lycopersicon esculentum* (tomato) lectin (5 mg / kg; *Vector Laboratories*, Burlingame, CA) to identify functional vasculature. After five minutes the mice were euthanized and the tumors were extracted and cut in half. One half of the tumor was fixed in 4% paraformaldehyde and embedded in paraffin. The other half of the tumor and five other extracted organs (liver, spleen, lungs, heart and abdominal skin) were weighed, minced with scissors, and suspended in sterile PBS. The minced organ suspension was serially diluted and plated on LB agar. Bacterial colonies were counted after overnight incubation at 37°C.

The embedded half of the tumor was cut into 5 μm serial sections. Biotinylated lectins bound to the vasculature were conjugated with Avidin (Vectastain Elite ABC kit) and subsequently visualized with diaminobenzidine (DAB) according to the manufacturer's recommendation. Successive sections were Brown-Hopps (Gram's stain) stained to identify gram-negative bacteria.

Statistics

Statistical significance was determined using ANOVA and Fisher's post-hoc test. The significance in the slope for the bacterial flux to adhesion comparison was determined using the *F* test for linear regression.

The following examples describe methods used to generate recombinant viruses for the expression of a protein that targets the interstitial matrix or the tumor vasculature. These examples are not meant to limit the invention in any way.

Example 5. Generation of recombinant HSV-1 amplicon vector containing the cDNA for collagenase.

To generate a recombinant HSV-1 amplicon vector expressing collagenase the cDNA for collagenase is inserted into pKSR2, an HSV-1 amplicon vector plasmid (Figure 6). The collagenase gene is expressed from the hCMV promoter and the fluorescent protein DsRed2 from the IE4/5 promoter. The amplicon plasmids contain the *pac* gene, which allows the transgenes to be packaged into the recombinant vectors (Figure 7).

The replication deficient amplicon vectors that express the collagenase are then packaged by transfecting a packaging cell line with the amplicon plasmid and a bacterial artificial chromosome (BAC) containing the entire 152-kb genome of HSV-1, but without the *pac* signals (Figure 8). Only the DNA in the amplicon plasmid is packaged, and the replication defective "amplicon vector" contains a 150-kb concatemer with multiple copies of the genes of interest (Figure 9). The amplicons express both DsRed2 (a red fluorescent probe used as a probe for imaging, Clonetech) and the collagenase (Figure 10). Methods for packaging are described in Saeki Y et al. (*Improved HSV-1 amplicon packaging system using ICP27-deleted, oversized HSV-1 BAC DNA*, In Viral vectors for gene therapy methods and protocols, Machida CA eds., p. 51-60, Humana Press Totowa, NJ 2003) and Sena-Esteves et al. (HSV-1 amplicon vectors--simplicity and versatility, Mol. Ther. 2: 9-15, 2000).

Example 6. *In vitro* determination of the ability of recombinant vectors to infect cells and express active collagenase.

Confirmation of the ability of the amplicon vectors to infect cancer cells and express active collagenase is carried out *in vitro*. Several human cancer cell lines (e.g., melanoma Mu89, soft tissue sarcoma HSTS26T, mammary carcinomas MDA-MB-231 and MDA-MB-361HK) are infected with amplicon vectors at various MOIs ($10^0 - 10^7$). DsRed2 expression is used to confirm infection of cells. Both the conditioned media and cell lysate are collected and a Western blot is performed to detect the presence of the collagenase. In addition, a collagenase activity assay on the conditioned media is performed to confirm that the enzyme is functional. Two types of assays for collagenase activity are performed and both are based on the detection of cleaved, fluorescently labeled collagen I. In one assay (from Chondrex), fluorescently labeled collagen monomers are incubated with the collagenase, cleaved products are separated from intact monomers (using the difference in denaturation temperature), and the amount of cleaved collagen is detected. In a second assay, fluorescently labeled collagen (Molecular Probes) is mixed with unlabeled collagen (Vitrogen – Cohesion Technologies) at a concentration of 2% and the mixture is polymerized to form a gel. The collagen gel is incubated with the conditioned media containing collagenase. After one hour, supernatant is collected and the amount of released (cleaved) collagen is determined. If necessary, the conditioned media of the infected cells can be concentrated (Centricon, 30kD). In addition, if necessary, for MMPs, the pro-collagenase can be activated prior to the activity assay using amino-phenyl mercuric acetate (APMA).

Example 7. *In vivo* determination of the ability of recombinant vectors to infect cells and express active collagenase.

An i*n vivo* assay is performed in order to determine if these amplicon vectors can infect cancer cells *in vivo* and express collagenase that will degrade interstitial fibrillar collagen. For this *in vivo* assay, tumors are grown in the dorsal skinfold chamber of SCID mice. Various amounts of amplicon vectors (ranging from 10^4 to 10^6 particles) are injected intratumorally in a volume of ~1 μL at a constant flow rate (0.5 μl/min). Prior to injection, and at various time points following injection (1hr, 1 day, 2 day, 4 days, 1 week), expression of DsRed2 by infected cells is visualized by two-photon microscopy. Simultaneously, second harmonic generation is used to image and quantify fibrillar collagen *in vivo*. See Brown et al., Nat Med 9: 796-800, 2003. The technique allows dynamic monitoring of both transgene expression and fibrillar collagen structure following infection with the amplicon vectors. In addition, immunofluorescence is used to semi-quantitatively determine levels of collagen I at the final time point. Changes in fibrillar collagen between the collagenase-expressing amplicons and control vectors are compared to determine if these amplicons can significantly degrade or alter the structure of intratumoral fibrillar collagen. Furthermore, ELISA assays (fluorokine E assay, R&D Systems) are performed to determine the levels of collagenase present in the tumor.

While high expression levels of particular MMPs have been related to increased tumor cell invasion and metastasis (Egeblad et al., Nat Rev Cancer, *2:* 161-174, 2002; Stamenkovic et al., J Pathol, *200:* 448-464, 2003), it is also likely that excessive degradation of fibrillar collagen disrupts the matrix and impairs tumor cell movement. The metastatic potential of MMP-overexpressing tumors is assessed as follows. Tumors are grown subcutaneously in the right hind foreleg of SCID mice and MMP expression is

induced when the tumors reach a diameter of ~5 mm. The primary tumor is then resected after one week and an autopsy is performed one month following tumor resection (Padera et al., Science, *296:* 1883-1886, 2002).

Example 8. Co-injection of a recombinant vector expressing collagenase and an oncolytic viral vector.

In addition, the effect of collagenase expression (and tumor fibrillar collagen modification) induced by these amplicon vectors on the distribution and efficacy of oncolytic viral therapy is determined. For this determination, the collagenase-expressing amplicon vectors are mixed with MGH-2 (an oncolytic HSV expressing EGFP for visualization) in various ratios. The mixtures of vectors are injected into tumors implanted in the dorsal skinfold chamber of SCID mice as described above. Two-photon microscopy is used to image cells infected by both amplicon vectors (expressing DsRed2) and MGH-2 (expressing EGFP), as well as the fibrillar collagen *in vivo*, at various time points following injection of the vectors. Assays are performed as described above to determine if there is any modification in collagen content or structure, and if there is an improvement in the distribution of oncolytic virus. Production of collagenase from the initial infection of cancer cells by amplicon vectors is expected to result in a degradation of collagen, which should allow the oncolytic MGH-2 particles produced from the initial round of infection to distribute throughout a greater volume of the tumor. Furthermore, the cells initially infected with both an amplicon vector and MGH-2 can produce more amplicon vector, which can subsequently infect different cancer cells, generating more collagenase. The amount of amplicon vector propagation depends on the initial ratio of amplicon vector to oncolytic vector. This assay is used to determine whether direct insertion of collagenase into MGH-2 improves its intratumoral distribution. Tumor size measurement can also be

used to determine if production of collagenase can ultimately improve efficacy of the oncolytic virus. An ELISA assay can be used as described above to measure the amount of the collagenase in the tumor.

Example 9. Insertion of collagenase cDNA directly into oncolytic vector.

To generate an oncolytic vector expressing collagenase, the collagenase cDNA is directly inserted into the oncolytic vector genome. The recombinant oncolytic vector is produced and the experiments described in Example 8 above are used to determine if the expression of collagenase improves the effectiveness of the oncolytic virus.

Example 10. Effect of Collagen on Viral Distribution in the Tumor Interstitium

In order to observe the effect of collagen on the distribution of viral vectors in the tumor interstitium, the following experiment was performed. Human melanoma Mu89 was implanted in the dorsal skinfold chamber of SCID mice. eGFP-labeled HSV was injected intratumorally at a constant flow rate of 0.5 μl/min. Two-photon microscopy was used to simultaneously image the fluorescent HSV particles and fibrillar collagen with second harmonic generation. The majority of HSV particles were excluded from regions rich in collagen fibers (Figure 11). The bulk of the HSV particles were excluded from regions that are rich in collagen. Some HSV particles were observed in between the collagen fibers. In the same tumor, the viral injection site was pretreated with bacterial collagenase for 1 hour. An increased spread of virus at the injection site was observed.

Example 11. Effect of *in vivo* transfection of MMPs with a replication-defective vector on interstitial transport

Collagen-degrading MMPs are delivered to the tumor using a replication-defective virus as a gene therapy vector. The cDNA of the selected MMP is subcloned into the genome of an HSV-1 amplicon vector plasmid. This vector expresses MMP from the hCMV promoter, eGFP from the IE4/6 promoter, and has an RFP-labeled tegument protein to highlight the viral particles. MDA-MB-361-HK is grown in the dorsal chamber and mammary fat pad, and Mu89 is grown in the dorsal chamber. To obtain a dose response the amount of virus is varied by three orders of magnitude. At several time points following injection the distribution of viral transgene expression in the tumor is observed with multi-photon laser scanning microscopy. Collagen content and fiber length is measured using second harmonic generation and transport studies are carried out in regions of both high and low viral transduction to facilitate comparison.

The methods of the invention are useful for inducing collagen degradation. This degradation breaks down the collagen fibers and facilitates the movement of molecules and viral particles through gaps in the collagen network. These gaps may allow large therapeutics to reach previously inaccessible regions in the tumor. The breakdown of the collagen network will likely be more effective at enhancing the distribution of replication-conditional viral particles that are significantly restricted in their movement by the narrow spaces between tumor cells. Replication-conditional viruses have an inherent mechanism for improving their distribution: tumor cell lysis that creates large gaps between tumor cells and facilitates viral movement. In addition the regulated expression of MMPs by tumor cells or fibroblasts throughout the tumor will likely lead to the more uniform distribution of therapeutic macromolecules than the intratumoral injection of the HSV-1 amplicon vector

expressing MMP. Finally, because of the close proximity of fibroblasts to collagen fibers, diffusion should be faster when MMPs are chronically expressed in fibroblasts than in tumor cells.

Other Embodiments

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in microbiology or related fields are intended to be within the scope of the invention.

What is claimed is: